AN EXPERIMENTAL INFECTION OF ALBINO MICE WITH *ENTAMEBA HISTOLYTICA*

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AN EXPERIMENTAL INFECTION OF ALBINO MICE WITH ENTAMEBA HISTOLYTICA

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Rees[1] has stated that one of the promising new fields in the diagnosis of Entameba histolytica infections is that of xenodiagnosis. At present, the most practical means of determining the presence of an Entameba histolytica infection is that of identification of the organism from a fecal specimen by a competent technician. This method, however, is not completely reliable. Attempts at diagnosis by means of immunological and cultural methods have thus far proven to be impractical. It appears at this time that there is no completely accurate means of determining the presence of the infection. However, if an animal could be found in which a one hundred per cent infection could be consistently established then a dependable method of diagnosis will have been found.

Using the technique of intracecal inoculation, this investigation was undertaken to establish an experimental infection in the albino mouse with Entameba histolytica, NIH 200 R strain. Special emphasis has been placed upon the percentage of mice infected and the tissue damage produced in the small and large intestine of the mouse by the amebae.

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[1] C. W. Rees, Problems in Amoebiasis (Springfield, Illinois: Thomas, 1955), p. 17.

CHAPTER II

HISTORY

Experimental work for the reliable diagnosis of Entameba histolytica infection has been directed towards four basic fronts. One path is that of identification of the amebae by examination of a fecal specimen by a competent technician. Another consists of attempts to grow organisms in culture from a fecal specimen. A third is an attempt to demonstrate antibodies present in the serum of the host which is infected with Entameba histolytica; and finally, the use of animal inoculation has been attempted.

Examination of a fecal specimen by a competent technician is the established and, at present, the most reliable method for demonstrating Entameba histolytica. This method, however, is not completely reliable and has some disadvantages. The method is time consuming and requires a highly trained technician. Moreover, the ability of technicians to identify the organism varies greatly from one individual to another.

There have been many attempts to culture Entameba histolytica from fecal specimens; but, until recently, the reliability of this method was not great because of the rich growth of organisms from the intestinal flora of the host.

Eyles[1] in an epidemiological study in the Memphis, Tennessee area was able to grow Entameba histolytica in culture from infected dogs when microscopic stool examination gave negative results. A commercially available medium consisting of an extract of egg yolk enriched with liver extract and rice powder has been developed by Balamuth and Sandza[2]. Nelson[3] has prepared an alcoholic extract medium which was found to be practical and effective as a medium for diagnosis for amebiasis and compared it with Bacto Endameba medium (Difco Laboratories). The former has the disadvantage that commensal ameba may be cultured; whereas in the latter, the medium is specific for Entameba histolytica. The Cleveland Collier medium[4] offers an attractive medium for the cultivation of the organisms in vitro from feces, provided a means

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[1]D. E. Eyles, F. E. Jones, J. R. Jumper, and V. P. Drinnon, "Amebic Infections in Dogs," Journal of Parasitology, XL (April, 1954), 163-166.

[2]W. Balamuth and J. G. Sandza, "Simple Standardized Culture Medium for Physiological Studies on Entameba histolytica," American Journal of Tropical Medicine and Hygiene, LVII (October, 1944), 161-162.

[3]E. C. Nelson, "Alcoholic Extracts Medium for the Diagnosis and Cultivation of Entameba histolytica," American Journal of Tropical Medicine, XXVII (September, 1947), 545-552.

[4]L. R. Cleveland and J. Collier, "Various Improvements in the Cultivation of Entameba histolytica," American Journal of Hygiene, XII (November, 1930), 606-613.

of inhibiting the yeast Blastocystis hominis can be found. Spingarn and Edelman[1] have been able to achieve up to seventy per cent isolation of Entameba histolytica from stools microscopically shown to be positive by the addition of twenty milligrams of streptomycin sulfate per milliliter to the cultures.

Attempts at immunodiagnosis have thus far proven to be impractical. Wagener[2] was able to demonstrate antibodies specific for Entameba histolytica in the serum of experimentally infected young and adult cats by means of a precipitin test. Craig[3] was able to demonstrate a hemolytic, cytolytic, and complement fixing substance in alcoholic extracts of Entameba histolytica. Craig[4] was able to show the presence of antibodies specific for Entameba histolytica in the blood stream of individuals infected with Entameba

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[1]C. L. Spingarn and M. H. Edelman, "Further Observations on the use of Streptomycin and Penicillin in the Cultivation of Entameba histolytica from Stools," American Journal of Tropical Medicine, I (May, 1952), 412-416.

[2]E. H. Wagener, "A Precipitin Test in Experimental Amoebic Dysentary in Cats," University of California Publications in Zoology, XXVI (January, 1924), 15-20.

[3]C. F. Craig, "Observations upon the Hemolytic, Cytolytic, and Complement Fixing Properties of Extracts of Entameba histolytica," American Journal of Tropical Medicine, VII (July, 1927), 225-240.

[4]C. F. Craig, "Complement Fixation in the Diagnosis of Infection with Entameba histolytica," American Journal of Tropical Medicine, VIII (January, 1928), 29-30.

histolytica but could not demonstrate antibodies in the serum of individuals recently cured of the infection. Magrath and Meleney[1] have reviewed work done with antigens prepared by alcoholic extraction. Apparently, the antigen is inadequate in that metabolic products from the bacterial associates mask the antigen. Rees et. al.[2] using monoxenic cultures of Entameba histolytica and organism t has prepared an antigen with a high degree of specificity with little or no involvement of the antigens donated by organism t. Organism t is thought to be a bacillus of the genus Clostridium. Kenney[3] using Rees's antigen has obtained positive results in eighty per cent of patients passing trophozoites. Recently, Cole and Kent[4] have been able to demonstrate antibodies produced in the rabbit, which are capable of temporarily immobilizing Entameba histolytica in vitro. At present, immunodiagnosis

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[1] T. B. Magrath and H. E. Meleney, "The Complement Fixation Reaction for Amebiasis: Comparative Tests performed by Two Laboratories," American Journal of Tropical Medicine, XX (March, 1940), 211-238.

[2] C. W. Rees, J. Boziecevich, L. V. Reardon, and F. Jones, "A Preliminary Note on the Complement Fixation Test for Amebiasis with Antigens Prepared from Entameba histolytica grown with a Single Species of Bacteria," American Journal of Tropical Medicine, XXII (November, 1942), 581-587.

[3] M. Kenney, "Micro-Kolmer Complement Fixation Test for Amebiasis," American Journal of Tropical Medicine, I (January, 1953), 717-730.

[4] B. A. Cole and J. F. Kent, "Immobilization of Entameba histolytica In Vitro by Antiserum produced in the Rabbit," Proceedings for the Society of Experimental Biology and Medicine, LIII (September, 1953), 811-815.

varies with the degree of infection, with the individual performing the test, and with the method of preparation of the antigen.

There is little information available in which animal inoculation has been used as a means of diagnosis for amebiasis. Dale and Dobell[1] having established fulminating infections with Entameba dysentariae, recommend the use of cats as a method for diagnosis of Entameba dysentariae infection. Investigations by Tobie[2], Carrera and Faust[3], and Taylor[4], of fulminating infections in the rabbit and guinea pig, using the technique of intracecal inoculation, has led to an interest in the use of these animals for the diagnosis of Entameba histolytica infections as suggested by Rees.

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[1]H. H. Dale and C. Dobell, "Experiments on the Therapeutics of Amoebic Dysentary," Journal of Pharmacology and Experimental Therapeutics, X (October, 1917), 399-459.

[2]J. E. Tobie, "An Experimental Infection of the Rabbit with Entameba histolytica," American Journal of Hygiene, XXIX (1949), 859-870.

[3]G. M. Carrera and E. C. Faust, "Susceptibility of the Guinea Pig to Entameba histolytica of Human Origin," American Journal of Tropical Medicine, XXX (1949), 647-667.

[4]D. J. Taylor, B. Highman, J. Greenberg, G. R. Coatney, "An Experimental Infection of the Guinea Pig with Entameba Histolytica," American Journal of Tropical Medicine, XXX (1950), 817-827.

It was demonstrated by Kessel[1] that the albino mouse is susceptable to infection with Entameba histolytica. Using oral feeding of cysts to adult animals, Kessel was able to establish an infection in one of six mice. It has been demonstrated by Keizo[2] that extreme liver damage occurs in the mouse following the injection of alcoholic extracts of Entameba histolytica.

There has been little work concerning infections in mice with Entameba histolytica reported since the investigation of Kessel. It should be of interest then, to determine the infectivity of albino mice to a known strain of Entameba histolytica.

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[1] J. F. Kessel, "An Experimental Infection of Rats and Mice with the Common Intestinal Amoebae of Man," University of California Publications in Zoology, XX (October, 1923), 409-440.

[2] H. Keizo, T. Koji, O. Suketaka, N. Yasuchika, and S. Toshisada, "Studies on the Toxic Effects of Entameba Histolytica on the Parenchymal Organs of Mice," Gunma Journal of Medical Science, I (June, 1955), 1-12.

CHAPTER III

MATERIALS AND METHODS

The organism used in this investigation was Entameba histolytica, NIH 200 R strain, obtained through the courtesy of Dr. Paul Thompson of Parke, Davis and Company, Detroit, Michigan. The amebae were cultured on Bacto Endameba medium (Difco Laboratories),[1] which were prepared so that a one centimeter butt was present at the bottom of the culture tube. The slant extended to the top of the culture tube. Sterile, screw cap, culture tubes fifteen centimeters long and two centimeters in diameter were used in this investigation. To the Bacto agar slants were added a sterile horse serum (Difco Laboratories) saline overlay containing twenty milligrams of streptomycin sulfate (Eli Lilly and Company) per milliliter. The streptomycin was added to inhibit the growth of the bacterial flora in the cultures.[2] The sterile horse serum saline overlay with streptomycin sulfate was prepared by adding four grams of sodium chloride crystals to five hundred millliliters of singly distilled water. The

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[1] Difco Laboratories. Difco Manual (Detroit, Michigan: Difco Laboratories Incorporated, 1963), p. 97.

[2] Spingarn and Edelman, Loc. cit.

physiological saline solution was autoclaved at one hundred and twenty-one degrees centigrade for fifteen minutes at fifteen pounds pressure (American Sterilizer Company). Streptomycin sulfate stock solution was prepared by adding one gram of sterile powdered streptomycin sulfate to ten milliliters of singly distilled water. Sixty milliliters of the sterile physiological saline solution was transferred to a sterile five hundred milliliter graduate cylinder and to this was added one ten milliliter ampoule of the sterile horse serum. From the streptomycin sulfate stock solution one and four-tenths of a milliliter was transferred with a one milliliter pipette into seventy milliliters of the horse serum saline overlay. To the above, a pinch of rice powder (Difco Laboratories) was added just before the cultures were ready to be used. Every seventy-two hours the amebae were transferred to three tubes of sterile culture media. Three cultures were used for each transfer because of the difficulty of maintaining viable cultures of the amebae. Transfer of the amebae from one culture to another was done by flaming the tops of the culture tubes in a microburner, inserting a sterile one milliliter pipette into the culture tube and withdrawing approximately one-tenth of a milliliter of the rice powder and horse serum saline overlay. Care was taken to avoid obtaining as much of the fluid portion of the culture

overlay as possible. Prior to transfer, the cultures were examined microscopically to determine the presence of the amebae. This was done in the following manner. A one milliliter pipette was inserted into the culture containing the amebae and approximately one-tenth of a milliliter of the rice powder was withdrawn from the culture and placed on a clean glass microscope slide. This live preparation was examined with a Spencer monocular compound microscope with the forty four power objective and a ten power ocular. The cultures were incubated at thirty seven degrees centigrade (Precision Scientific Company).

Upon receiving the amebae from Parke, Davis and Company, three permanent slide preparations were prepared from cultures containing the amebae and stained with Delafield's hematoxylin[1] to determine the morphology of the amebae. The permanent slides were prepared by fixing the amebae with Schaudinn's fixative for one hour.[2] Mayer's egg albumin was spread over a clean microscope slide by rubbing it on with a clean little finger.[3] About two-tenths

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[1] P. Grey, Handbook of Basic Microtechnique (New York: McGraw Hill Book Company, 1958), p. 83-84.

[2] E. Gurr, Staining Practical and Theoretical (Baltimore: Williams and Eilkins Company, 1962), p. 537.

[3] Ibid.

of a milliliter of the rice powder and horse serum saline overlay were withdrawn from the culture with a one milliliter pipette without agitation of the culture. The contents were allowed to flow onto the area where the Mayer's egg albumin had been spread. The two-tenths of a milliliter of the rice powder, horse serum saline overlay mixture was allowed to partially evaporate. After fixing in Schaudinn's solution for one hour the preparations were washed in a seventy per cent iodine ethyl alcohol (three per cent iodine in seventy per cent ethyl alcohol) solution for five minutes to remove mercuric chloride crystals. They were then transferred to ninety five and seventy per cent ethyl alcohol solutions for five minutes each, and following this were placed under a cold water tap in a Koplin jar and water was allowed to gently flow over the slides for ten minutes. The amebae were stained in Delafield's hematoxylin for four or five minutes, and then washed with cold water from the tap for ten minutes. The slides were then transferred through seventy, ninety five per cent and absolute ethyl alcohol for five minutes each. After clearing in toluene for ten minutes, the preparations were mounted in HSR (Hartmann Leddon Company) synthetic resin. Care was taken to insure that the slides did not dry at any time during the procedure.

Estimation of the number of trophozoites of Entameba

histolytica in the cultures was made with an improved Neubauer Ultraplane Spotlite hemacytometer (Scientific Products Company). The cultures were agitated by rotating them between the palms of the hands until an apparent homogeneous suspension of the rice powder was obtained. Immediately, the contents of the horse serum saline overlay containing the amebae, which was about one cubic centimeter, was poured from the culture into a watchglass fifteen centimeters in diameter and pipetted with a one milliliter pipette onto the two counting chambers of the hemacytometer. Sufficient culture was added to cover the ruled area of the counting chamber without the excess flowing into the moat around the counting chambers. The hemacytometer coverslip was placed on the counting chamber and the estimate made. Eight squares were selected at random by drawing numbers from a hat. Two readings were made for each culture. Estimates were made by using intermediate squares each of which contained one tenth of a cubic millimeter volume. Only the amebae touching the upper left corner and lines of the intermediate squares were counted.[1, 2] The number of trophozoites

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[1] M. Paulson, "An Accurate Method for the Numerical Determination of Entameba Histolytica In Vitro and its Possible use with other Intestinal Protozoa," American Journal of Tropical Medicine, XII (September, 1932), 387-389.

[2] E. L. Levenson and McFate, Clinical Laboratory Diagnosis (Lea and Peafiger, 1959), p. 721-739.

in one cubic centimeter of culture was estimated by multiplying the number of trophozoites counted in each intermediate square by ten to give the number of trophozoites per cubic millimeter and this number was multiplied by one thousand to give the number of trophozoites per cubic centimeter. All estimates of cultures to be used were made from selected cultures and a count of the ameba was made not more than six hours before inoculation of the mice. The cultures were selected by sampling them to determine if the amabae were abundant in the cultures.

The above cultures were to be used to inject the contained amebae into the mice. To determine if the amebae population was adequate, the contents of one-tenth of a milliliter of the culture was pipetted with a one milliliter pipette onto the two counting chambers of the hemacytometer and the number of trophozoites in one cubic centimeter of the culture was estimated as described previously. The cultures were again agitated and all the contents poured into a watchglass fifteen centimeters in diameter. Approximately one cubic centimeter of the culture was withdrawn from the watchglass into a Hypak five cubic centimeter disposable syringe without a needle. A number twenty-six guage hypodermic needle was then attached to the syringe and the contents of the syringe was shaken vigorously until a homogeneous

suspension of the rice powder was obtained. The number of trophozoites in one cubic centimeter of culture was estimated by using the hemacytometer. The results are shown in Table I (see Appendix).

Sterilization procedures consisted of autoclaving the Bacto Endameba medium at one hundred and twenty-one degrees centigrade at fifteen pounds of pressure for fifteen minutes. One, five, and ten milliliter pipettes, a one five hundred milliliter graduate cylinder and two screw cap culture tubes containing rice powder were sterilized in a hot air oven (Precision Scientific Company) at one hundred and sixty degrees centigrade for two hours. The pipettes were stored in sterile steel containers. All transfer of materials from one container to another was preceeded by flaming the tops of the tubes or containers in a microburner.

Four groups, each consisting of ten albino mice at five, seven, nine, and eleven weeks of age with a minimum of three control mice for each group were used in this investigation. The mice were albino mice which were obtained from Drake University, Des Moines, Iowa. At all times males and females were kept apart to avoid pregnancy as a variable. Five week old mice were weaned at least five days prior to inoculation with Entameba histolytica, NIH 200 R strain. The purpose of this was to eliminate any milk factor in the

diet and to keep all mice as homogeneous as possible with respect to diet. All mice were fed on Purina Laboratory Chow (Formula: C-5001). Twenty-four hours prior to the operation and also twenty-four hours postoperatively food was withdrawn from the mice but water was available at all times. No attempt was made to determine the parasite mix of the mice other than the presence or absence of Entameba histolytica. To determine the presence of Entameba histolytica, a fecal pellet from all mice of each group was cultivated in Bacto Endameba medium containing the rice powder and the horse serum saline overlay. This was designated as Culture I. Sodium Pentabarbitol (Abbott) was used as the anesthetic, with the amount to be intraperitoneally injected, based upon the body weight of the individual mouse. The Sodium Pentabarbitol solution contained twenty-five hundredths of a milligram per milliliter of the powder in seventy per cent aqueous ethyl alcohol solution. It was found by the investigator that twenty-five hundredths of a milligram per milliliter of the powder was sufficient to anesthetize a twenty-five thousands of a kilogram mouse for forty-five minutes without the animal waking up. If for a twenty-five thousandths kilogram mouse one milliliter of Sodium Pentabarbitol solution was sufficient to anesthetize the animal for forty-five minutes; then, for a mouse whose

weight is known, it should be possible to calculate the amount of Sodium Pentabarbitol to be administered to any mouse of a known weight. The weight of each mouse was determined not more than three days prior to the operation by weighing them on a single pan balance (E. H. Sargent Company).

Sodium Pentabarbitol was administered with a one cubic centimeter disposable syringe with a twenty-six gauge needle. Care was taken to swab the injection site both before and after administration of the anesthetic with seventy per cent ethyl alcohol. The purpose of this was to clean the injection site and to mat down the hair so as not to get hair into the incision. After anesthesia had been induced, the mouse was fastened to the operating board which consisted of a dissecting pan and rubber bands to bind the mouse. When upon pinching the hind toes of the mouse with hemostatic forceps, no reflex of withdrawing the leg was initiated, anesthesia had been induced deep enough so that the operation could be performed. The abdomen was again swabbed with seventy per cent ethyl alcohol and the incision through the skin was made midventrally with sterile, nickel plated, straight, fine-pointed scissors. The incision through the skin was at first made transversely for one or two millimeters about five-tenths of one centimeter anteriorly to the hind legs. Perpendicularly to the transverse cut, the

incision was extended along the midventral line for one centimeter. A sterile carbon steel forty millimeter scalpel was used to make a similar incision along the linea alba. No transverse incision was made. Bleeding was minimal. Sterile glass rods approximately three inches long and curved at the end sufficiently to grasp the cecum were introduced into the abdominal cavity and the cecum was withdrawn from the body cavity into the operative wound. At this time the inoculum consisting of the amebae was prepared by transferring the cultures into a fifteen centimeter watchglass and drawing the amebae into the syringe as has been described previously. A twenty six gauge hypodermic needle was attached to the syringe and approximately 40,000 trophozoites of Entameba histolytica were injected into the cecum. The hypodermic needle was inserted into the last one centimeter of the cecum by grasping the cecum with hemostatic forceps and gently pushing the needle through the cecal wall. Care was observed to insure that the needle did not puncture the opposite side of the cecal wall from where it was introduced. The hypodermic needle was slowly removed from the cecum to retard bleeding. The injection site on the cecum was swabbed in two per cent aqueous iodine solution to sterilize the injection site and to kill any amebae which might have leaked out of the needle puncture onto the cecal serosa. The cecum

was reinserted into the body cavity. Using a square knot, the body wall incision was sutured with 000 catgut. The wound was again swabbed with the two per cent aqueous iodine solution and the animal was allowed to recover. As stated previously, postoperative care consisted of restricting food to the mice for twenty-four hours.

Aseptic procedures were used throughout the operation. All instruments were sterilized in five per cent aqueous Roccal (Winthrop-Stearn) solution for thirty minutes and then transferred to seventy per cent ethyl alcohol solution. The working area and the operating board were thoroughly washed with Roccal. The hands of the investigator were washed with Borax hand soap (United States Borax and Chemical Corporation) before each operation was performed. No gloves were worn during the operation.

Fecal examination of the five and seven week old mice was made by attempting to culture the amebae in Bacto Endameba medium with the horse serum saline overlay and a pinch of rice powder. In general, the time of fecal examination depended upon the state of health of the individual mouse. If the mouse was lying in its cage unable to walk, the animal was sacrificed; or if the mouse began to display a sick condition and it would have to remain overnight before the cage was checked again, the mouse was sacrificed. The

purpose of this was to always have freshly killed mice for fecal examination. The mice were sacrificed by placing a fifteen centimeter nail horozontally behind the occipital bone of the skull and pulling the tail of the mouse.

Autopsy of the mice was performed in the following manner. A midline incision was made with the fine-pointed, dissecting, scissors about five tenths of a centimeter anterior to the hind legs and extending to the xiphoid process of the sternum. At the anterior portion of the incision, two transverse incisions were made on each side following the last rib around to the vertebral column. At the posterior portion of the incision, two cuts were made on each side through the body wall and extended around to the vertebral column. The two body wall flaps were pushed to the lateral side of the mouse and pinned with steel straight pins exposing the viscera. The viscera were examined macroscopically with a binocular dissecting scope (Bausch and Lomb) for lesions. The lesions were noted as white spots on the viscera and were recorded as present or absent.

In the nine and eleven week old mice, fecal examination was made as above but microscopic fecal examination was also made. Microscopic fecal examination consisted of excising a one centimeter length of cecum and squeezing the contents of the cecum out with a pair of blunt tweezers. The contents was placed on a slide upon which two drops of

physiological saline which had been incubated at thirty-seven degrees centigrade had been added. The material was comminuted with the tweezers and the preparation was stained with D'Antoni's iodine.[1] Two culture examinations were made from all mice postoperatively. One designated as Culture II consisted of inoculating Bacto Endameba cultures with an excised section of about one centimeter of the cecum. Culture III consisted of inoculating the cultures with an excised one centimeter section of lower colon. The contents of the intestinal segments also were incubated in the same cultures. All cultures were examined three days after inoculation to determine the presence of the ameba.

The following precautions were taken to dispose of all contaminated materials. All injected mice and excised parts were wrapped in two sheets of newspaper and bound with rubber bands. They were again wrapped with two sheets of paper and bound with rubber bands. The bound packages were then discarded into a waste can and ultimately the packages were incinerated. Contaminated cultures were autoclaved at one hundred and twenty-one degrees centigrade for fifteen minutes at fifteen pounds of pressure. The culture tubes were then washed with FAB detergent (Proctor and Gamble) rinsed and dried. Contaminated cages and instruments were

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[1]F. M. Spencer and L. S. Monroe, Color Atlas of Intestinal Parasites (Lea and Feabiger, 1961), p. 16.

boiled in water at one hundred degrees centigrade for twenty minutes, dried and stored. Debris from the cages was wrapped as stated and thrown into the waste can. The area of operation was thoroughly swabbed down with five per cent aqueous Roccal solution.

If lesions were present macroscopically, permanent tissue sections were made by fixing a section of the intestine with Zenker's fixative, embedding in paraffin, and using standard histological technique, staining with Delafield's hematoxylin and counterstaining with eosin.[1] The sections were then studied with a Spencer binocular compound microscope with the ten power and forty-four power objectives and a ten power ocular.

One eleven week old mouse was injected intracecally with approximately 40,000 trophozoites of Entameba histolytica, using the procedure described earlier, to determine if the amebae were viable when introduced into the cecum of the mouse. One hour after inoculation of the amebae, the cecum was excised by removing it with fine-pointed dissecting scissors and squeezing its contents onto a glass microscope slide containing physiological saline which had been warmed to thirty seven degrees centigrade. The contents of the cecum was comminuted with the hemostatic forceps and the two

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[1] P. Grey, op. cit., p. 214-221.

wet mount preparations were examined with a Spencer monocular compound microscope using the forty-four power objective.

To determine if the amebae could be recovered after being injected into the cecum, six mice, thirteen weeks old, were divided into three groups and treated in the following manner. The first group, consisting of three mice, was injected with Entameba histolytica, containing about 40,000 trophozoites as estimated by hemacytometer, and sacrificed. The cecum was excised ten minutes later with the dissecting scissors and the contents were squeezed onto a clean glass microscope slide and comminuted with physiological saline which had been heated to thirty seven degrees centigrade. The preparation was observed with a Spencer monocular compound microscope with the forty-four power objective to determine if the amebae were present or absent. The second group consisting of one mouse was treated in exactly the same manner and was sacrificed at the end of one minute following the inoculation with the amebae. The third group consisting of two mice was treated as above except the mice were sacrificed immediately after the inoculum of approximately 40,000 trophozoites had been injected into the cecum. The procedures used with each group were the same as those used with all previous mice except for those specially noted above.

CHAPTER IV

RESULTS AND INTERPRETATION OF DATA

The experimental infection of albino mice with Entameba histolytica, NIH 200 R strain was not accomplished with the techniques used in this investigation.

A summary of the data for the five, seven, nine, and eleven week old mice is presented in Tables II, III, IV, and V respectively (see Appendix). In reference to these tables, it can be seen that in all groups at no time was it possible to recover Entameba histolytica from any of the mice. In the five and seven week old mice, two cultures of Bacto Endameba medium were used in an attempt to recover the amebae and in nine and eleven week old mice, two culture examinations were supplemented with microscopic fecal examination. The culture examination using Bacto Endameba medium in this investigation was the same procedure used by Nelson[1] for recovering Entameba histolytica from human stool specimens. Culture examination and microscopic fecal examination were considered to be sufficient to recover the amebae from the mice. As will be shown, the absence of positive results was probably due to the amebae not being present in the large intestine of the mice at the time attempts were made to

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[1]Nelson, Loc. cit.

recover them. In reference to Table I (see Appendix), it can be seen that the amebae survived the passage with little or no reduction in their numbers through the disposable Hypak syringe to which a twenty-six gauge needle was attached. This would indicate that the same number of amebae which were counted with the hemacytometer were introduced into the cecae of the mice when injected. As noted in Table VI, it can be seen that the amebae were demonstrable only when the cecum was excised immediately after the amebae were injected. In spite of the limited work shown in Table VI (see Appendix), it is suggested that some factor is causing the amebae to lyse shortly after they are introduced into the cecum. The possibility of the amebae being voided with the feces after injection into the mice does not seem feasible. Food was restricted to the mice for twenty-four hours prior to the operation and for twenty-four hours postoperatively. During this time little defecation occurred. In those mice dying soon after the operation, fecal examination was carried out from feces taken from an excised portion of the lower colon of the mouse. It appears unlikely that the amebae could have been present in the mice during this period and not have been recovered. The possibility of the dilution of the amebae after injection into the cecae of the mice to such an extent that it would be difficult to recover them also appears

unlikely. Forty thousand trophozoites of Entameba histolytica were injected into the cecae of the mice. In those mice, as shown in Table VI, in which recovery of the amebae was attempted soon after their injection, it is unlikely that dilution of the amebae could occur to such a great extent in the length of time prescribed, before examination, such that the amebae could not be recovered. A factor other than lysis of the amebae might be a metabolic factor or osmotic "shock" due to the transfer of the amebae from the Bacto Endameba cultures directly into the cecal environment. Kessel[1] was able to infect one mouse by feeding Entameba dysentariae cysts orally. It is possible the amebae had time to acclimate to the intestinal environment before or during excystation. In this investigation, however, the trophozoites of Entameba histolytica were directly injected into the cecae of the mice. It appears that lysis of the amebae is also a possibility due to the secretion of the large number of cecal microorganisms present or to a secretion of the intestinal mucosa.

In those mice dying from an involvement of the duodenum, duodenum and cecum or for which the cause of death was unknown, symptoms were of a characteristic pattern. Typically, the mice would become listless and refuse to eat.

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[1]Kessel, Loc. cit.

As symptoms progressed the back of the animal would become arched and the head would be held low to the bottom of the cage. Eventually the mice would be unable to walk. No diarrhea or dysentary were noticed in any of the mice.

As noted in Tables II and IV (see Appendix), it can be seen that in the five and nine week old mice, few had to be sacrificed at the termination of the experiment. In the nine week old group this was due to two factors: the high incidence of mice in the experimental group dying from involvement of the duodenum (thirty per cent) and the large number of deaths to which no cause could be attributed (thirty per cent). In the five week old group the main cause of death involved both the cecum and the duodenum (fifty per cent). This did not appear in any of the other groups. As can be seen in Tables II, III, IV, and V, the majority of the mice lived for five days before they were sacrificed. It is from the group for which the cause of death was unknown that most of the microscopic permanent tissue sections were prepared. This is the only group of mice which could have died from the presence of the amebae. The incidence of involvement of the duodenum only was twenty to thirty per cent in all groups. It is believed, therefore, that in those mice dying from an involvement of the duodenum, and the duodenum and cecum, that possibly this may have been a result

of bacterial invasion of these areas during the operative procedure. Permanent tissue sections were made from one mouse showing this condition to determine if the amebae were present in these areas of the intestine.

Macroscopically, involvement of the duodenum and cecum showed a yellow to green discoloration of the tissue. If the above condition was in an advanced stage, the tissue would assume a jelly-like consistency and eventually the duodenal area could not be distinguished.

Microscopic examination of the duodenum showed that no amebae were present in the tissue. Figures I and II show a normal section of duodenum from a nine week old mouse. Figures III and IV show a diseased section of duodenum from a nine week old experimental mouse. It can be seen in Figures III and IV that there is general degeneration of the villi with complete deterioration and sloughing off of the mucosal cells into the lumen of the duodenum. The submucosa shows no vessels or connective tissue cells characteristic of the normal tissue. General necrosis may extend to the inner layer of circular muscles. There are no crater-like lesions or amebae demonstrable. In Tables II, III, IV, and V, those mice classified as having lesions macroscopically, were not found to have lesions. The white flecks noted by the dissecting scope were found microscopically to be lymphatic tissue along the large intestine.

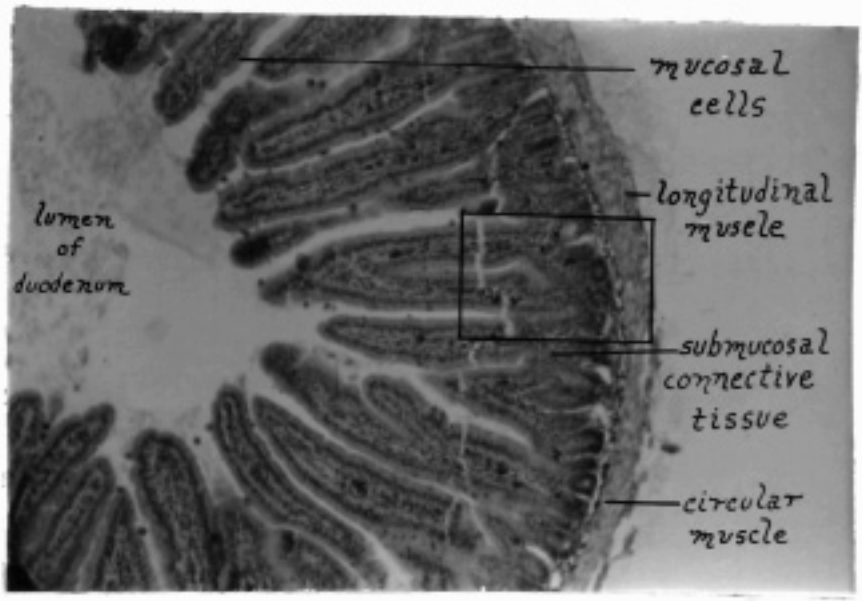

Figure 1. Delafield's Hematoxylin and Eosin counterstained tissue sections of duodenum of nine week old control mouse (10x).

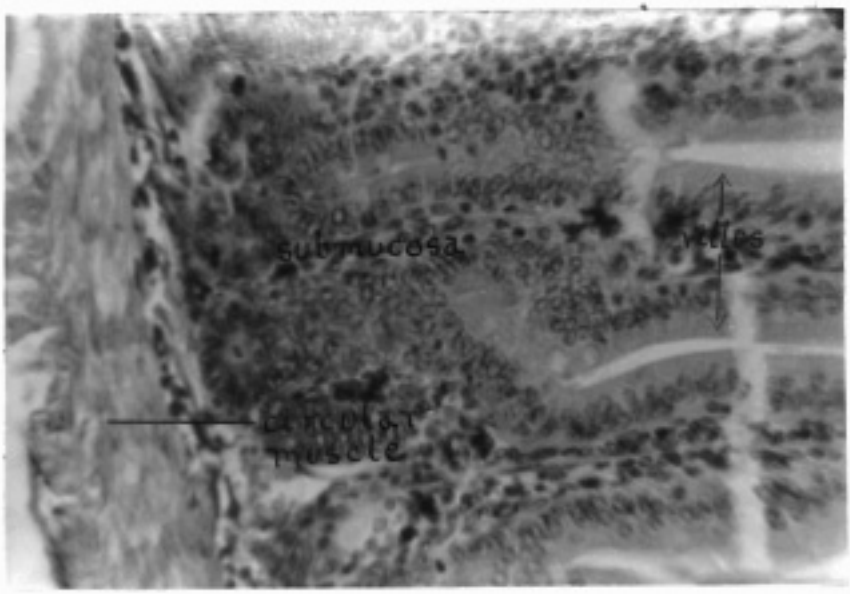

Figure 2. Section of Figure 1 (44x).

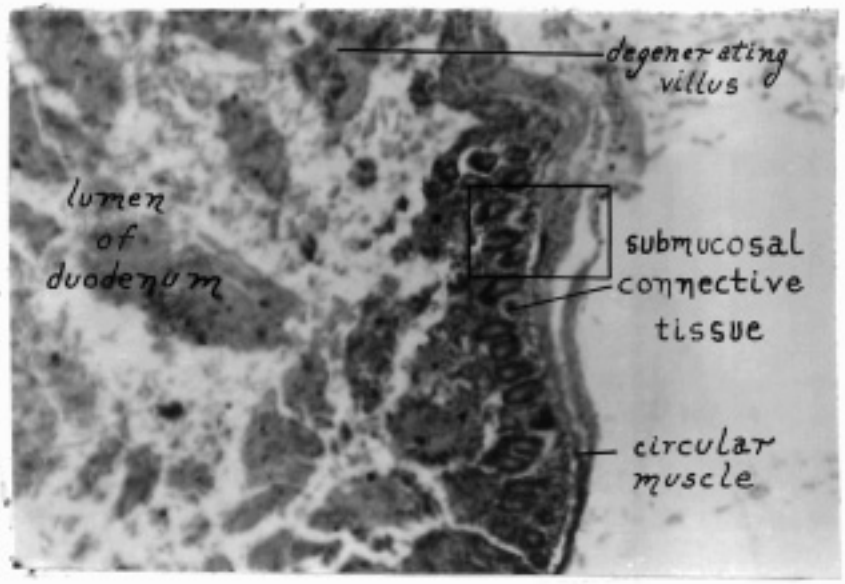

Figure 3. Delafield's Hematoxylin and Eosin counterstained tissue sections of duodenum of nine week old experimental mouse (10x).

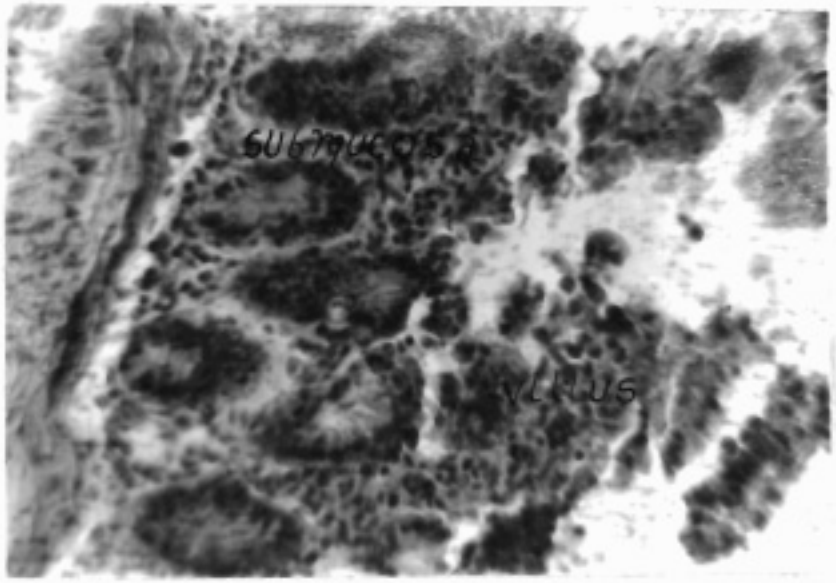

Figure 4. Section of Figure 3 (44x).

From Tables II, III, IV, and V, it is apparent that those mice which were sacrificed, were done at the end of the five days after the inoculation of the mice with the amebae. Those mice for which the cause of their death was unknown lived for a maximum of two days. Those mice showing involvement of the duodenum lived for a maximum of one day in the seven, nine, and eleven week old groups. In the five week old group, the mice lived for three days. Those mice showing involvement of the cecum as well as the duodenum lived for a maximum of two days. As can be seen there is a consistent pattern between the number of days the mice survived and the death of the mice.

From the results obtained in this investigation, it appears the laboratory mouse is refractory to invasion of Entameba histolytica with the procedures used in this study. Other techniques such as the use of cysts, other strains of Entameba histolytica, other strains of mice, modifications of the diet, and the use of sterile animals may reveal more favorable results.

CHAPTER V

SUMMARY

Immunological, cultural, and microscopic techniques have been used as a means of diagnosis for amebiasis.

In this investigation, an experimental infection of the laboratory mouse was attempted using Entameba histolytica, NIH 200 R strain. Approximately 40,000 trophozoites as estimated by hemacytometer were injected intracecally into the mice. In five and seven week old mice, attempts to recover the amebae were made by inoculating sterile Bacto Endameba cultures consisting of a horse serum saline overlay and a pinch of rice powder, with a one centimeter section from the cecum and colon. The same procedure was carried out in the nine and eleven week old mice except microscopic fecal examination was also made. Permanent tissue sections stained with Delafield's hematoxylin and counterstained with eosin were made of the colon, cecum, and duodenum of seven of those animals which were classified as positive for lesions.

From the results of this study it is suggested that the albino mouse is not susceptable to Entameba histolytica, NIH 200 R strain, with the techniques used in this investigation. Both cultural and microscopic fecal examination gave negative results in all groups. Attempts to demonstrate

the amebae in the mice by preparing permanent tissue section gave negative results. It is suggested that the amebae may be lysed after being injected into the cecum of the mice.

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APPENDIX

TABLE I

COMPARISON OF HEMACYTOMETER ESTIMATES OF THE NUMBER OF TROPHOZOITES OF ENTAMEBA HISTOLYTICA BEFORE AND AFTER TRANSFER THROUGH A FIVE CUBIC CENTIMETER SYRINGE AND A TWENTY SIX GUAGE HYPODERMIC NEEDLE

Culture Number	Before Transfer	After Transfer
1	35,800	44,400
2	48,500	70,150
3	33,000	29,700
4	36,500	40,200
5	37,900	36,200
6	36,500	39,200
7	35,200	32,200
8	66,500	59,500
9	33,900	29,000
10	27,200	27,000

TABLE II

SUMMARY OF DATA OF FIVE WEEK OLD MICE INOCULATED WITH ENTAMEBA HISTOLYTICA

Group	Animal Number	Presence of Lesions	Presence of Ameba			Microscopic Fecal Examination	Number of Days Mice Survived	Reason for Death
			Culture I	Culture II	Culture III			
Experimental	1	+	-	-	-	- - - - - - -	5	sacrificed
	2	+	-	-	-	- - - - - - -	5	sacrificed
	3	+	-	-	-	- - - - - - -	3	duodenum involv.
	4	+	-	-	-	- - - - - - -	2	duodenum involv.
	5	+	-	-	-	- - - - - - -	2	sacrificed
	6	+	-	-	-	- - - - - - -	2	duodenum-cecum
	7	-	-	-	-	- - - - - - -	1	duodenum-cecum
	8	-	-	-	-	- - - - - - -	1	duodenum-cecum
	9	+	-	-	-	- - - - - - -	1	duodenum-cecum
	10	-	-	-	-	- - - - - - -	1	duodenum-cecum
Control	11	-	-	-	-	- - - - - - -	5	sacrificed
	12	-	-	-	-	- - - - - - -	5	sacrificed
	13	-	-	-	-	- - - - - - -	1	operation

NOTE: Culture I refers to cultivation of one fecal pellet in Bacto Endameba medium prior to inoculation of the mice with E. histolytica. Culture II refers to inoculation of the culture with a section of cecum and Culture III refers to inoculation of cultures with a section of lower colon.

TABLE III

SUMMARY OF DATA OF SEVEN WEEK OLD MICE INOCULATED WITH ENTAMEBA HISTOLYTICA

Group	Animal Number	Presence of Lesions	Presence of Ameba			Microscopic Fecal Examination	Number of Days Mice Survived	Reason for Death
			Culture I	Culture II	Culture III			
Experimental	1	-	-	-	-	- - - - - -	5	sacrificed
	2	-	-	-	-	- - - - - -	5	sacrificed
	3	+	-	-	-	- - - - - -	5	sacrificed
	4	+	-	-	-	- - - - - -	5	sacrificed
	5	-	-	-	-	- - - - - -	5	sacrificed
	6	+	-	-	-	- - - - - -	5	sacrificed
	7	+	-	-	-	- - - - - -	0	unknown
	8	+	-	-	-	- - - - - -	1	duodenum involv.
	9	-	-	-	-	- - - - - -	1	sacrificed
	10	-	-	-	-	- - - - - -	1	duodenum involv.
Control	11	-	-	-	-	- - - - - -	0	operation
	12	-	-	-	-	- - - - - -	5	sacrificed
	13	-	-	-	-	- - - - - -	5	sacrificed

NOTE: Culture I refers to cultivation of one fecal pellet in Bacto Endameba medium prior to inoculation of the mice with E. histolytica. Culture II refers to inoculation of the cultures with a section of cecum and Culture III refers to inoculation of cultures with a section of lower colon.

TABLE IV

SUMMARY OF DATA OF NINE WEEK OLE MICE INOCULATED WITH ENTAMEBA HISTOLYTICA

Group	Animal Number	Presence of Lesions	Presence of Ameba			Microscopic Fecal Examination	Number of Days Mice Survived	Reason for Death
			Culture I	Culture II	Culture III			
Experimental	1	-	-	-	-	-	5	sacrificed
	2	-	-	-	-	-	5	sacrificed
	3	-	-	-	-	-	5	sacrificed
	4	+	-	-	-	-	5	sacrificed
	5	-	-	-	-	-	2	unknown
	6	-	-	-	-	-	2	unknown
	7	+	-	-	-	-	2	unknown
	8	+	-	-	-	-	1	duodenum involv.
	9	+	-	-	-	-	1	duodenum involv.
	10	+	-	-	-	-	1	duodenum involv.
Control	11	-	-	-	-	-	5	sacrificed
	12	-	-	-	-	-	5	sacrificed
	13	-	-	-	-	-	5	sacrificed

NOTE: Culture I refers to cultivation of one fecal pellet in Bacto Endameba medium prior to inoculation of the mice with E. histolytica. Culture II refers to inoculation of the cultures with a section of cecum and Culture III refers to inoculation of cultures with a section of lower colon.

TABLE V

SUMMARY OF DATA OF ELEVEN WEEK OLD MICE INOCULATED WITH ENTAMEBA HISTOLYTICA

Group	Animal Number	Presence of Lesions	Presence of Ameba			Microscopic Fecal Examination	Number of Days Mice Survived	Reason for Death
			Culture I	Culture II	Culture III			
Experimental	1	-	-	-	-	-	1	unknown
	2	+	-	-	-	-	1	duodenum involv.
	3	+	-	-	-	-	1	duodenum involv.
	4	-	-	-	-	-	6	sacrificed
	5	- - -	-	- - -	- - -	- - -	0	operation
	6	-	-	-	-	-	6	sacrificed
	7	+	-	-	-	-	6	sacrificed
	8	+	-	-	-	-	6	sacrificed
	9	+	-	-	-	-	6	sacrificed
	10	-	-	-	-	-	6	sacrificed
Control	11	-	-	-	-	-	6	sacrificed
	12	-	-	-	-	-	6	sacrificed
	13	-	-	-	-	-	6	sacrificed

NOTE: Culture I refers to cultivation of one fecal pellet in Bacto Endameba medium prior to inoculation of the mice with E. histolytica. Culture II refers to inoculation of the culture with a section of cecum and Culture III refers to inoculation of cultures with a section of lower colon.

TABLE VI

RECOVERY OF *ENTAMEBA HISTOLYTICA* AFTER INJECTION OF 40,000 TROPHOZOITES INTO THE CECUM OF MICE

Animal Number	Time After Inoculation Before Attempts to Recover the Ameba in Minutes	Microscopic Examination
1	60.0	-
2	11.0	-
3	10.0	-
4	10.0	-
5	1.0	-
6	0.5	-
7	0.5	+